

Several Log Increase in Therapeutic Transgene Delivery by Distinct Adeno-Associated Viral Serotype Vectors

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Received for publication November 1, 2000; accepted November 1, 2000

We previously demonstrated that AAV vectors carrying human and canine factor IX (FIX) cDNA can infect, stably persist, and secrete functional human and canine FIX following direct intramuscular injection. In an attempt to improve FIX protein secretion for eventual therapeutic use, we set out to determine if alteration of the AAV capsid would affect skeletal muscle transduction and factor IX secretion. Two reasons to pursue this question were (1) the persistence of high-titer neutralizing antibody (NAB) to AAV2 and (2) our previous study that supported a restricted tropism of muscle fibroblasts to AAV2 transduction. Using an identical CMV/canine factor IX (cFIX) expression cassette, we cross-packaged this genome into virions generated from each of the five AAV serotypes. In a dose-response assay, equivalent amounts of rAAV/cFIX serotypes were tested *in vitro* and *in vivo*. In tissue culture cells, FIX antigen levels secreted into the supernatant varied depending on the AAV serotype used; type 2 transduced maximally, with serotypes 3, 1, 5, and 4, respectively, expressing lower levels. However, when the same viruses were tested *in vivo* using immunodeficient NOD/SCID animals, we obtained surprisingly different results. While the time to onset of detectable serum levels appeared the same for all serotypes, types 1, 3, and 5 produced 100- to 1000-fold more cFIX than type 2. In fact, 12 weeks after transduction, type 1 continued to express levels of cFIX on average at 80 µg/ml followed by type 5 (6.52 µg/ml), type 3 (3.27 µg/ml), type 4 (258 ng/ml), and finally type 2 (90 ng/ml). Coagulant activity of cFIX as measured by a PTT supported the circulating levels measured by ELISA demonstrating that the secreted protein was functional, and RT-PCR of injected tissue correlated with the serotype-specific transduction data. In summary, we found significant differences in cFIX expression upon introducing various rAAV serotypes into mouse muscle. These data have direct bearing on the design of AAV gene therapy clinical trials for hemophilia and should also extend to most therapeutic transgenes.

INTRODUCTION

Recombinant adeno-associated virus (rAAV) is considered a safe and promising vector for human gene therapy. AAV is nonpathogenic, infects both dividing and nondividing cells, can establish latency, and persists in tissue such as skeletal muscle, retina, liver, brain, and vasculature (1). A variety of therapeutic proteins including factor IX (FIX) are expressed via simple intramuscular injection of recombinant AAV (2–4). Of the six AAV serotypes, serotype type 2 (AAV2) is best characterized and therefore predominantly used in gene transfer studies (5). Here we report that AAV vector types 1, 3, 4, and 5 transduces skeletal muscle more efficiently and secretes canine

FIX (cFIX) at levels two- to three logs greater than rAAV2. Thus, without modifying gene expression cassettes, the capsid composition of the majority of AAV serotype appears to effect vector transduction significantly enough to produce higher than therapeutic levels of circulating FIX.

Six serotypes of AAV have been cloned and sequenced with five of the six having divergent amino acid sequences. Serotypes 1 and 6 share >99% amino acid homology in their capsid proteins and sequence analysis supports recombination even between serotypes 1 and 2 (6, 7, 14). Comparison of the serotype capsid amino acid sequences suggests that types 1, 2, and 3 share homology across the three capsids in accord with heparan sulfate binding (8). In contrast, AAV types 4 and 5 share less homology across the capsid region and neither type 4 nor type 5 binding is competed by soluble heparan sulfate, suggesting that they use distinct receptors (9, 10).

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Hemophilia B is the clinical manifestation of inheritance factor IX deficiency (11). We and others demonstrated that AAV2 targeting skeletal muscle produces functional factor IX in large animal models but at less than therapeutic levels (2, 12). Recent reports in brains suggest that AAV serotypes 4 and 5 have different host cell tropisms *in vivo* (13). With respect to skeletal muscle transduction, AAV2 preferentially infects slow muscle fiber groups, indicating a restricted tropism in muscle (15), whereas type 1 has been shown to have a marked increase in muscle transduction (14).

METHODS

rAAV production. All rAAV vectors were produced, purified, and titered as previously described (2). The rAAV/green fluorescence protein (GFP) or cFIX DNA template utilized for encapsidation in AAV1, 2, 3, and 4 were derived from plasmid pSub201 as previously described (2). rAAV5 virus was generated after subcloning the EGFP and cFIX cDNAs into plasmid p7D05 containing two type 5 ITRs (10) via *Bgl*II digestion. The AAV5 vector plasmid p7D05 and helper plasmid pAAV5 were generously supplied by Drs. Chiorini and Kotin (NHLBI, NIH, Bethesda, MD). All other AAV packaging plasmids were supplied through the UNC Gene Therapy Vector Core and described by Rabinowitz and Samulski (submitted).

Canine FIX assays. Canine FIX antigen and activity assays (ELISA and aPTT) were performed as previously described (2) with minor modifications. Purified canine FIX (Affinity Biological Inc., Hamilton, Canada) was added to pooled NOD/SCID mice plasma and serial dilutions were made. This was used as standard for ELISA and aPTT. Plasma from experimental animals was diluted 4- to 400-fold for both ELISA and aPTT assays. Plasma from animals that were injected with AAV/EGFP was used as control.

Animal care and procedures. NOD/SCID animals (Jackson Labs, Bar Harbor, ME) were maintained and treated in accordance with the Animal Care and Use Committee of UNC Chapel Hill. One hundred microliters of PBS containing 1.5×10^{10} rAAV/GFP or 2.5×10^{11} rAAV/cFIX virions was directly injected into the gastrocnemius muscle in 4- to 5-week-old NOD/SCID mice. The mice were bled via retroorbital plexus every 4 weeks postinjection. Eight weeks after AAV injections, the mice receiving rAAV/GFP were anesthetized by 2.5% avertin and perfused through cardiac puncture with 4% paraformaldehyde (Sigma) and 0.25% glutaraldehyde (Sigma). The gastrocnemius muscles with injected rAAV (marked with coinjection of carbon particles) were harvested and sliced at 25- μ m intervals. The muscle specimens were observed by fluorescent microscopy (Model DM-IRB, Leica, Germany). Twelve weeks after AAV injection, mice in each experimental group receiving serotyped rAAV/cFIX were sacrificed, and the skeletal muscle was harvested and processed. RNA was isolated using a Qiagen Rneasy kit (Qiagen, Germany), following the manufacturer's protocol. RT-PCR was performed as described previously (2).

RESULTS

To investigate all the AAV serotypes for efficient muscle transduction, we generated high-titer rAAV type 1, 2, 3, 4, and 5 viruses expressing humanized GFP or canine factor IX by transient triple-plasmid transfection as previously described (2). An AAV type 2 vector plasmid carrying either a CMV-driven EGFP or cFIX gene was used with the appropriate helper plasmid (see Methods) to cross-package and generate rAAV/EGFP or rAAV/cFIX 1, 2, 3, and 4 vectors. These recombinant vectors differ only in capsid structure (Rabinowitz and Samulski, submit-

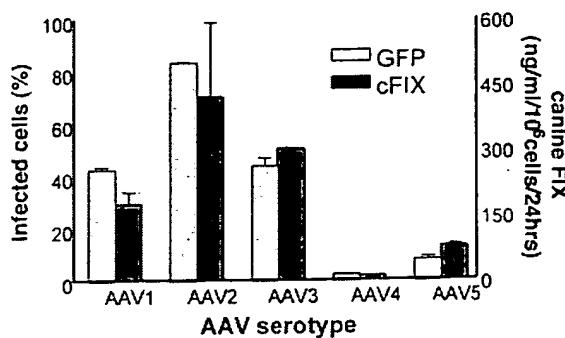


FIG. 1. 293 cells were infected with either rAAV1-to-S/GFP or rAAV1-to-S/cFIX at a m.o.i. of 10^3 vector genomes/cell. Quantitation of each virus preparation (vector genomes/ml) by dot-blot hybridization was performed prior to each experiment and all vectors were prepared, stored, and used identically. Adenovirus type 5 (wtAd5) was added at a m.o.i. of 2/cell, and rAAV1-to-S/GFP-infected cells were analyzed by flow cytometry 24 h postinfection. The percentage of GFP-positive cells was determined, and cFIX antigen in the cell supernatant was measured by ELISA. No detectable fluorescence or cFIX was observed in mock-infected controls (Ad5 only, data not shown).

ted). To generate rAAV5, we subcloned the EGFP or cFIX expression cassette into p7D05 that contains rAAV5 ITR elements. This vector was cotransfected with pAAV5 (containing the rep and cap genes of AAV5) as previously described (10) to generate rAAV5/EGFP and rAAV5/cFIX. All viruses were purified and particle numbers determined by dot-blot assay more than three times.

Serotype-Specific Transduction *In Vitro*

Each recombinant serotype was tested for transduction by infecting the human-derived 293 cell line in duplicate using identical numbers of viral particles. Serotype 2 EGFP and cFIX vectors produced the highest transduction levels (Fig. 1). These observations were followed by AAV types 3, 1, and 5 and finally serotype 4. The low level of rAAV4/EGFP fluorescence and rAAV4/cFIX levels secreted were consistent with previous reports demonstrating the inefficient transduction of human vs nonhuman primate cells (9). In addition, the difference observed with type 2 transduction on 293 cells may be related to the fact that this cell has been characterized for expressing cell-surface heparan sulfate which is employed by AAV2 as one of its cell receptors (10). At present, none of the receptor molecules used by the other serotypes has been described. In these studies, we used vector particle number to control for m.o.i. Because both GFP and cFIX vector preps transduced equally for each respective serotype (Fig. 1), we concluded that transduction differences observed between serotypes were not due to variation from vector production and purification. To confirm these observations, specific serotypes were produced more than once and retested for *in vitro* and *in vivo* transduction with similar results.



FIG. 2. *In vivo* transduction of skeletal muscle using rAAV serotypes. rAAV/EGFP types 1–5 were injected into the hindlimbs of 4-week-old NOD/SCID mice at 1.5×10^{10} genomes/animal, respectively. Mice were sacrificed 8 weeks postinjection; the injected skeletal muscle was processed and photographed (magnification 200 \times). (A) rAAV1/GFP-injected muscle, (B) rAAV2/GFP-injected muscle, (C) rAAV5/GFP-injected muscle. To control for background fluorescence, a 500-nm band-width pass filter was used.

Serotype-Specific Transduction *In Vivo*

Direct intramuscular injection of rAAV2 transduces skeletal muscle cells and persists long-term in animal models(2). To investigate *in vivo* transduction efficiency, the five different serotypes of rAAV/GFP and cFIX were directly injected into gastrocnemius muscle of 4- to 5-week-old NOD/SCID mice (see Methods). Skeletal muscle fluorescence for GFP transduction measured at 8 weeks was positive for all five rAAV serotypes with AAV1 and AAV5 displaying the most intense and widespread staining. The GFP staining patterns suggest that type 1 and 5 vectors diffused across the length of the entire muscle analyzed unlike the “patchwork” appearance typically observed with AAV2 (Fig. 2).

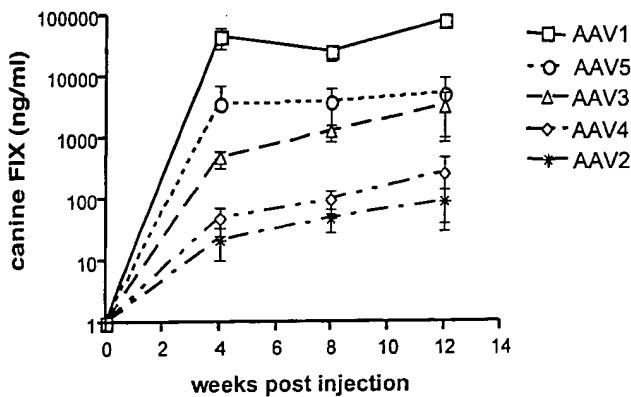


FIG. 3. Plasma antigen levels of canine FIX in NOD/SCID mice after intramuscular injection of rAAV serotypes. Mice received 2.5×10^{11} vector genomes of each AAV/cFIX serotype. A volume of 100 μ l virus was injected into the gastrocnemius muscle ($n = 5$ mice for each serotype tested). Mice were bled at monthly intervals and the plasma was measured by ELISA for cFIX antigen. Plasma from animals injected with AAV/EGFP was used as control.

Based on these results, we then tested the five serotype AAV/canine FIX vectors in skeletal muscle. 2.5×10^{11} genome particles of rAAV/cFIX 1, 2, 3, 4, and 5 were injected into the hindlimbs of NOD/SCID mice ($n = 5$ for each experimental group). Plasma was tested at monthly intervals for canine factor IX using an ELISA specific for canine factor IX (see Methods). As shown in Fig. 3, we detected canine factor IX in all experimental animals. No canine factor IX antigen was detected from plasma samples taken from control animals receiving equivalent doses of serotyped AAV/EGFP (data not shown). As expected and consistent with the GFP studies, cFIX antigen levels in mice receiving rAAV types 1, 3, 4, and 5 were higher than those in the rAAV2/cFIX mice (Fig. 3). The amount of canine factor IX in the plasma of mice receiving types 1, 3, and 5 was unexpected. Levels higher than 100 and 10 μ g/ml of cFIX were detected in the mice receiving rAAV types 1 and 5, respectively. Even serotype 3, which by sequence is most similar to type 2, expressed cFIX levels up to 5.8 μ g/ml. This represents 10^3 and 10^2 increases of cFIX, respectively, when compared to mice receiving rAAV2/cFIX.

Vector-Transduced Canine FIX Is Biologically Active

The supraphysiologic levels of cFIX allowed us to dilute the endogenous mouse FIX and test the coagulant activity of transgene protein. The aPTT of (normal) control mice was significantly prolonged at 5-fold and greater dilutions of plasma (Table 1). In marked contrast, 80- to 160-fold dilutions of rAAV1/cFIX mouse plasma were required before the aPTT time was prolonged. To quantify the aPTT activity in terms of absolute amounts of protein, we established a standard aPTT curve by the addition of purified canine factor IX to control mouse plasma (data not shown). Based on these results, cFIX antigen and activity levels were consistent and indicated

TABLE I
aPTT Clotting Times of the Plasma of rAAV1/cFIX-Treated Mice

Dilution factor	Control plasma	Clotting time(s)					Purified cFIX in mice plasma
		1629	1630	1885	1886	1887	
1:5	96.6	ND	ND	ND	ND	ND	—
1:10	113.1	51.3	ND	ND	ND	ND	—
1:20	ND	71.9	65.1	ND	ND	ND	86.1(900ng/ml)
1:40	ND	84.8	80.9	79.9	88.4	82.6	95.6(450ng/ml)
1:80	ND	99.15	94.7	89.6	108.9	104.4	105.1(225ng/ml)
1:160	ND	137.3	113.1	109.6	122.6	123.1	114.9(112.5ng/ml)

Note. Plasma was collected using heparin-coated microhemocrit capillary (Fisher); heparin was inactivated using Dade Hepzyme (Dade Behring Marburg GmbH, Germany) following the manufacturer's protocol. Plasma from the rAAV1/GFP-treated mouse (control plasma) was diluted 5-to-10-fold for aPTT assay. Plasma from the rAAV1/cFIX-treated mice was diluted 10-to-160-fold. Purified cFIX was added to control plasma and diluted serially. All results are expressed as averages of duplicate samples performed in two separate experiments.

that the canine factor IX secreted was fully functional (Fig. 4).

RT-PCR Analysis of Vector-Transduced Muscle

Molecular analysis of rAAV/cFIX mRNA in mouse skeletal muscle was performed from each serotype-specific transduced group. At 12 weeks postinjection, animals were sacrificed, gastrocnemius muscle was isolated, and mRNA was prepared for RT-PCR. Based on semi-quantitative PCR (Fig. 5), the amount of detectable transcript was consistent with the plasma levels of factor IX (Fig. 3) and the extent of vector spread observed with GFP expression (Fig. 2).

DISCUSSION

In this study, we characterized the transduction of all five AAV serotypes as vectors carrying an identical expression cassette for GFP and cFIX transgenes. All serotype-specific vectors produced similar vector yields (10^{12} – 10^{13} particles/ml) and were positive for transduction both *in vitro* and *in vivo*. However, level of transduction *in vivo* did not mirror *in vitro* results. In fact, we observed *in vivo* expression levels of cFIX that were 100 to 1000 times higher than the commonly used serotype 2 vectors. The cFIX protein expressed from all vectors was biologically active based on aPTT assays, and RT-PCR supported serotype-specific transduction levels. Based on our studies, AAV type 1 vectors appeared to be the most efficient in muscle transduction, with expression levels of cFIX that averaged 80 µg/ml followed by type 5 (6.52 µg/ml), type 3 (3.27 µg/ml), type 4 (258 ng/ml), and finally type 2 (90 ng/ml). A previous report comparing AAV type 1 and AAV type 2 vectors in muscle using different reporter genes and breeds of mice (14) also described an increased vector transduction for type 1 albeit at lower levels.

One primary reason for investigating different AAV serotype vectors was aimed at evading existing immune responses to AAV serotype 2. Antibody to AAV2 in the

normal population ranges between 50 and 96%. Of these individuals, 18–67% carry neutralizing antibodies. We have found in both mice and dogs that neutralizing antibodies are long lasting and remain at a high titer after primary exposure to AAV2, making readministration of this serotype ineffective (Chao and Walsh, unpublished data). From these animal studies, the existing immune response to type 2 vector may have a significant impact on *in vivo* gene transfer in humans. The lack of cross-reactivity among neutralizing antibodies of different rAAV serotypes in mice (Li and Samulski, unpublished data) suggests that vector readministration using non-AAV type 2 vectors may bypass this problem. Although we feel confident that the different serotype vectors will provide alternative approaches to readministration, more important is the fact that we obtained higher than therapeutic levels of FIX using non-type 2 AAV vectors.

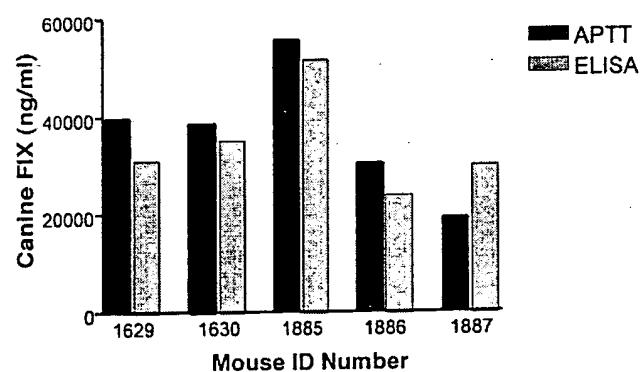


FIG. 4. Procoagulant activity of canine FIX in serotype 1-treated animals. ELISA antigen and aPTT activities were measured in plasma samples from AAV1-treated animals. Results are expressed as averages of samples performed in duplicate for each animal tested. Purified cFIX was added to NOD/SCID mouse plasma and serial dilutions were made to generate a standard curve of aPTT activity. Plasma samples of rAAV1-treated animals were diluted 10-to-160-fold. cFIX values (ng/ml) were calculated based on the aPTT standard curve. aPTT activity of plasma from AAV/EGFP-injected animals was used as control.

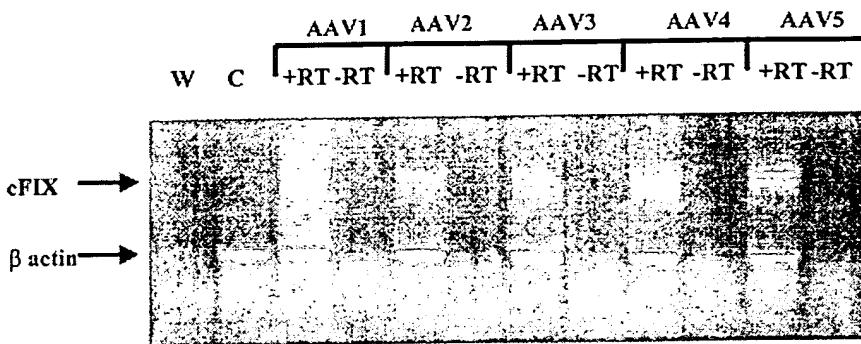


FIG. 5. RNA expression of AAV/cFIX types 1–5 in skeletal muscle. Total cellular RNA was isolated from skeletal muscle and RT-PCR performed as described under Methods. Primers specific for the cFIX transgene amplified a 1000-bp band. β -Actin (250-bp band) was used as a control. RNA samples were treated with DNase; each sample was treated with (+RT) and without reverse transcriptase (−RT) to control for DNA contamination. Water (W) and RNA isolated from control animal muscle (C) are shown at the left. Eight hundred nanograms of RNA was used in each RT-PCR.

To our knowledge, this is the first report obtaining significantly higher than physiological levels of cFIX expression without modifying the expression cassette. In fact, the use of identical expression cassettes in these various serotypes strongly supports viral tropism as the primary reason for increased transduction. These data were supported by serotype-specific GFP transduction in mouse muscle. At present, all animal studies using AAV vectors have consistently yielded long-term transgene expression. In our hands, we have positive expression approaching 3 years in the hemophilic dog model. This strongly suggests that repeated administration may not be necessary if therapeutic levels can be obtained with initial doses. In addition, our data in this mouse model strongly support using serotype 1 AAV vectors for human clinical trials. One concern with testing these serotype-specific vectors in mouse models is that these results may be species-specific. However, this has not been the case with serotype 2 vectors, which have successfully transduced mice, rabbit, dog, and monkey. At present, we are testing large-animal models to determine if these serotype-specific vectors will perform equivalently in all models (mouse, dog, and primate).

Finally, the results of this study suggest that AAV vector-mediated gene transfer is substantially improved by using naturally occurring serotypes as vectors. This approach may be useful for the treatment of a wide variety of inherited diseases.

ACKNOWLEDGMENTS

The author thanks Paul Monahan for helpful discussions and Larry Arnold for technical assistance and flow cytometry analysis. This work was supported in part by Grants NIH DK54419 and HL48347 to R. J. Samulski and C. E. Walsh. In addition, C. E. Walsh is a recipient of the Lucille Markey Charitable Trust Award.

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